# Locomotor conditioning by amphetamine requires cyclin-dependent kinase 5 signaling in the nucleus accumbens

Article (Accepted Version)

# Locomotor conditioning by amphetamine requires cyclin-dependent kinase 5 signaling in the nucleus accumbens

Bryan F Singer[†1], Nichole M Neugebauer[2], Justin Forneris[2], Kelli R Rodvelt[2], Dongdong Li[2], Nancy Bubula[2], Paul Vezina[*,1,2]

[1]Committee on Neurobiology
[2]Department of Psychiatry and Behavioral Neuroscience
The University of Chicago, Chicago, IL, USA

*****, Correspondence: Paul Vezina
Department of Psychiatry and Behavioral Neuroscience
The University of Chicago
5841 S. Maryland Avenue, MC 3077
Chicago, IL 60637
TEL: 01-773-702-2890
FAX: 01-773-702-0857
E-MAIL: pvezina@yoda.bsd.uchicago.edu

†, Current address: Department of Psychology
University of Michigan
Ann Arbor, MI, USA

**ABSTRACT**

Intermittent systemic exposure to psychostimulants such as amphetamine leads to several forms of long-lasting behavioral plasticity including non-associative sensitization and associative conditioning. In the nucleus accumbens (NAcc), the protein serine/threonine kinase cyclin-dependent kinase 5 (Cdk5) and its phosphorylation target, the guanine-nucleotide exchange factor kalirin-7 (Kal7), may contribute to the neuroadaptations underlying each of these forms of plasticity. Pharmacological inhibition of Cdk5 in the NAcc prevents the increases in dendritic spine density in this site and enhances the locomotor sensitization normally observed following repeated cocaine. Mice lacking the *Kal7* gene display similar phenotypes suggesting that locomotor sensitization and increased NAcc spine density need not be positively correlated. As increases in spine density may relate to the formation of associative memories and both Cdk5 and Kal7 regulate the generation of spines following repeated drug exposure, we hypothesized that either inhibiting Cdk5 or preventing its phosphorylation of Kal7 in the NAcc may prevent the induction of drug conditioning. In the present experiments, blockade in rats of NAcc Cdk5 activity with roscovitine (40 nmol/0.5µl/side) prior to each of 4 injections of amphetamine (1.5 mg/kg; i.p.) prevented the accrual of contextual locomotor conditioning but spared the induction of locomotor sensitization as revealed on tests conducted one week later. Similarly, transient viral expression in the NAcc exclusively during amphetamine exposure of a threonine-alanine mutant form of Kal7 [mKal7(T1590A)] that is not phosphorylated by Cdk5 also prevented the accrual of contextual conditioning and spared the induction of sensitization. These results indicate that Cdk5 phosphorylation of Kal7 in the NAcc is

necessary for the formation of context-drug associations potentially through the modulation of dendritic spine dynamics in this site.

**Keywords:** conditioning, dendritic spines, psychostimulants, learning, memory, roscovitine, sensitization

## INTRODUCTION

Repeated intermittent amphetamine exposure can lead to several forms of behavioral plasticity including associative conditioning and non-associative sensitization. The formation of associative memories can link contextual stimuli to unconditioned drug effects allowing these cues to elicit drug-like excitatory conditioned responses as demonstrated by the locomotor activating effects of amphetamine and contexts previously paired with amphetamine (Stewart, 1992; Stewart and Vezina, 1988). In the case of sensitization, drug-evoked behavioral and neurochemical responses become exaggerated with successive infusions of the drug (Vezina, 2004). This form of plasticity accrues independent of association formation as demonstrated by the ability of amphetamine infusions into the ventral tegmental area (VTA) to produce sensitization in the absence of drug conditioning (Singer *et al*, 2009; Vezina and Stewart, 1990). Although associative conditioning and non-associative sensitization reflect distinct processes, drug-paired and drug-unpaired environments can come to control the expression of sensitized responding (Anagnostaras and Robinson, 1996; Anagnostaras *et al*, 2002; Stewart and Vezina, 1988, 1991; Wang and Hsiao, 2003). As both of these forms of plasticity are known to regulate drug-related behaviors and have been linked, separately and together, to addiction vulnerability in humans and animal models (Vezina and Leyton, 2009; Leyton and Vezina, 2013), it is important to elucidate their underlying neuronal mechanisms.

Drugs of abuse are typically administered in the presence of a large number of salient environmental stimuli, providing ample opportunity for the formation of drug-stimulus associations and the possibility for these associations to subsequently

influence responding. Morphological changes in dendritic spines have long been thought to underlie aspects of this type of memory storage and have been observed following learning, the induction of long-term potentiation, and behavioral enrichment (Geinisman *et al*, 2001; Lamprecht and LeDoux, 2004; Leuner et al, 2003). In the nucleus accumbens (NAcc), exposure to sensitizing regimens of systemic amphetamine injections produces long lasting increases in dendritic spine density (Robinson and Kolb, 1997, 1999). Considering that these are not observed following repeated infusions of amphetamine into the VTA, it is likely that they reflect associative drug conditioning rather than non-associative sensitization (Singer *et al*, 2009). Consistent with this possibility, Marie *et al* (2012) showed that the development of cocaine CPP correlates with dendritic spine density in the NAcc. Thus, preventing these increases in NAcc dendritic spine density normally observed in rats exposed to systemic amphetamine would be predicted to inhibit the development of conditioning while preserving the induction of sensitization. This reasoning provided the rationale for the present experiments to investigate the contribution to the induction of conditioning of proteins known to regulate dendritic spine dynamics.

Two such proteins, the proline-directed serine/threonine kinase cyclin-dependent kinase 5 (Cdk5) and its phosphorylation target, the guanine-nucleotide exchange factor kalirin-7 (Kal7), are known amongst other actions to regulate cytoskeletal stability related to dendritic spine formation and retraction (Penzes and Jones, 2008; Xie *et al*, 2007; Xin *et al*, 2008) and have been implicated in drug-induced spine proliferation in the NAcc. As predicted, pharmacological inhibition of Cdk5 in the NAcc blocks cocaine-induced increases in dendritic spine density in this site (Norrholm *et al*, 2003) but

enhances the induction of locomotor sensitization (Bibb *et al*, 2001; Taylor *et al*, 2007). Similarly, mutant mice lacking the *Kal7* gene display enhanced locomotor sensitization but without the increases in dendritic spine density normally observed in the NAcc following cocaine exposure (Kiraly *et al*, 2010). However, the link between the actions of Cdk5 and Kal7 in the NAcc and the development of drug conditioning remains unclear.

Although its effect in the NAcc has not yet been assessed and no experiments have yet been conducted with amphetamine, pharmacological inhibition of Cdk5 in the lateral septum and hippocampus was reported to block the acquisition of fear conditioning (Fischer *et al*, 2002) and when applied to the basolateral amygdala, to prevent the acquisition of cocaine CPP (Li *et al*, 2010). On the other hand, strategies using transgenic mice or viral-mediated gene transfer to target the NAcc have yielded conflicting results regarding the functions of Cdk5 and Kal7 in Pavlovian and instrumental conditioning. Mice with either Cdk5 knocked out (Hawasli et al, 2007) or with Cdk5 activity increased (Fischer et al, 2005) both show enhanced contextual fear conditioning. Relative to control conditions, selective Cdk5 knock-out in the NAcc has been reported to produce both decreased and increased acquisition of cocaine CPP (Benavides *et al*, 2007) while decreased acquisition of cocaine CPP has been shown in Kal7 knock-out mice (Kiraly *et al*, 2010). Reducing Kal7 in the NAcc with lentiviral delivery of Kal7 shRNA decreased incentive motivation but had no effect on the acquisition of cocaine self-administration (Wang *et al*, 2013) while Cdk5 knock-out mice show similarly unaffected acquisition of instrumental responding but enhanced incentive motivation (Benavides et al, 2007). One possible explanation for these conflicting results may lie in the fact that unlike in pharmacology studies, the Cdk5 and Kal7

manipulation strategies used in the latter experiments did not distinguish between acquisition and expression of conditioning, making the results obtained difficult to interpret. As with sensitization (Vezina, 2004), different neuronal mechanisms underlie the acquisition and expression of excitatory conditioning (Aujla and Benninger, 2004; Banasikowski *et al*, 2010; Cervo and Samanin, 1995) and these may be differentially affected by changes in Cdk5 and Kal7.

The present experiments assessed the contribution of Cdk5 and Kal7 in the NAcc to the induction of amphetamine-induced locomotor conditioning and sensitization. The approach used targeted induction specifically by pharmacologically inhibiting Cdk5 or using a transient viral infection system to express exclusively during amphetamine exposure a threonine-alanine mutant form of Kal7 (mKal7) that is not phosphorylated by Cdk5. Our results indicate that Cdk5 phosphorylation of Kal7 in the NAcc is necessary for the induction of excitatory contextual drug conditioning.

## MATERIALS AND METHODS

### Animals

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, WI) weighing 250-275g on arrival were used. Rats were individually housed in a reverse cycle room (12-h light/12-h dark; lights on at 2000 hours) with food and water available *ad libitum*. All procedures were performed during the dark phase of the light cycle. Following a 4-5 day acclimation period, all rats were anesthetized with a mix of ketamine (100 mg/kg, IP) and xylazine (10 mg/kg, IP), placed in a stereotaxic instrument with the incisor bar positioned 5.0 mm above the interaural line, and implanted with 22 gauge chronic

bilateral guide cannulae angled at 10$^{o}$ to the vertical and aimed at the NAcc shell (A/P, +3.4; M/L, ± 0.8; DV, -7.5mm from bregma and skull; as per the angled brain atlas of Pellegrino *et al*, 1979) with tips positioned 1 mm (for the roscovitine experiment) or 4 mm (for the HSV-mKal7 experiment) above the final injection site. The NAcc shell was targeted because previous studies of the effects of NAcc roscovitine examined this subnucleus (Norrholm *et al*, 2003; Taylor *et* al, 2007) and it is uniquely innervated by the ventral hippocampus, a structure known to process contextual information (Moses *et al*, 2002). The cannulae (Plastics One, Roanoke, VA) were imbedded in a dental cement cap secured by six screws fastened to the skull. After surgery, 28 gauge obturators were placed into the guide cannulae (either flush for the HSV-mKal7 experiment or protruding 1mm beyond the guide cannula tips for the roscovitine experiment) and rats were returned to their home cage for 10-14 days of recovery. All surgical procedures were conducted using aseptic techniques according to an approved Institutional Animal Care and Use Committee protocol.

**Locomotor Testing Chambers**

A bank of 8 open field activity boxes (Med Associates, St. Albans, VT) was used to measure locomotor responding to saline and amphetamine. Each open field (43.2 X 43.2 X 30.5 cm) was constructed of acrylic walls, a wire mesh floor, a removable Plexiglas top, and was fitted with a 16 X 16 horizontal grid of infrared sensors positioned 3.5 cm above the floor. Separate interruptions of photocell beams were detected as ambulatory counts and recorded via an electrical interface by a computer situated in an adjacent room using Med Associates Open Field Activity Software (SOF-811).

**Effect of Inhibiting Cdk5 in the NAcc on the Induction of Locomotor Conditioning and Sensitization by Amphetamine**

In this experiment, rats were subjected to three phases: drug exposure, withdrawal, and testing.

The exposure phase used a discrimination learning paradigm that consisted of four 3-day conditioning blocks (Table 1). Injections were given on the first two days of each block (the first in the open field and the second in the home cage); rats were left undisturbed in the home cage on the third. For each block, rats in two groups (Paired-Veh and Paired-Ros) were administered amphetamine (1.5 mg/kg, IP) in the open field preceded 30 minutes earlier by bilateral infusion into the NAcc of vehicle (Veh; 0.5µl/side) or the Cdk5 inhibitor roscovitine (Ros; 40 nmol/0.5µl/side) and locomotor activity was recorded for two hours. The following day, these rats were administered saline (1.0 ml/kg, IP) in the home cage preceded by NAcc obturator movements to mimic NAcc microinjections (thereby reducing the total number of actual microinjections into tissue). Rats in two additional groups (Unpaired-Veh and Unpaired-Ros) were administered the same injections but in the reverse order: saline with obturator movements in the open field and amphetamine preceded by vehicle or roscovitine in the home cage. Rats in two final groups (Control-Veh and Control-Ros) received saline in both environments preceded either by NAcc vehicle, roscovitine, or obturator movements. No differences in open field locomotion were observed during the exposure phase between controls administered roscovitine in the open field or the home cage. The data for these animals was therefore combined.

Following the 12 days of exposure (4 X 3-day blocks), rats were afforded a 1-week withdrawal period during which they were left undisturbed in the home cage. Rats were then tested for conditioned locomotion for 1-hour in the open field following a saline injection (1.0 ml/kg, IP) or for locomotor sensitization for 2-hours following an amphetamine injection (1.0 mg/kg, IP). Roscovitine was not administered before either test. Thus, 12 separate groups of rats were tested in this experiment: 6 for conditioned locomotion and 6 for locomotor sensitization (Table 1). A lower dose of amphetamine was used on the test for sensitization because when administered repeatedly, doses of amphetamine used to sensitize locomotion can also lead to sensitization of competing stereotypic behaviors that sometimes occlude progressive enhancements in locomotion during exposure (Stewart and Vezina, 1987; Combag *et al*, 1999).

**Drugs and Microinjections**

S(+)-amphetamine sulfate (Sigma-Aldrich Inc., Saint Louis, MO) was dissolved in sterile saline. The Cdk5 inhibitor (R)-roscovitine (Enzo Life Sciences Inc., Plymouth Meeting, PA) was dissolved in 1XPBS/50% DMSO vehicle. Doses refer to the weight of the salt and were selected based on effective doses administered in previous reports (Bibb *et al*, 2001; Singer *et al*, 2009; Taylor *et al*, 2007).

Bilateral NAcc roscovitine and vehicle microinjections were performed in freely moving rats using 1µl syringes (Hamilton, Reno, NV) connected to injection cannulae (28 gauge) via PE20 tubing. Injectors were inserted 1 mm beyond the guide cannula tips and 0.5µl of the solution was simultaneously infused into each hemisphere over a 30-second period. Following a diffusion time of 1 minute, injectors were removed and obturators replaced.

**Effect of Transiently Expressing mKal7 in the NAcc on the Induction of Locomotor Conditioning and Sensitization by Amphetamine**

In this experiment, rats were subjected to four phases: viral infection, drug exposure, withdrawal, and testing.

Replication-deficient herpes simplex virus (HSV) vectors were chosen to express exclusively during amphetamine exposure a serine-alanine mutant form of Kal7 (mKal7) in the NAcc that is not phosphorylated by Cdk5 as these viral vectors produce transient expression of the transgene lasting 4-5 days (Carlezon and Neve, 2003; Loweth *et al*, 2010; Neve *et al*, 1997; Singer *et al*, 2010). This allowed for selective disruption of Cdk5-Kal7 signaling in the NAcc only during the acquisition of conditioning. As expected, no evidence for mKal7 expression remained 8 days post infection, well before the tests for expression of locomotor conditioning and sensitization. Control rats were administered NAcc infusions of 1X PBS vehicle or HSV vectors to transiently express GFP (Mock).

The exposure phase used a discrimination learning paradigm that consisted of four 2-session conditioning days (Table 2) beginning the day after viral infection. This compacted drug exposure phase was designed to accommodate the transient infection afforded by the HSV vectors. On each day, rats were administered an injection in the open field in one session and in the home cage in the other. These sessions were separated by five hours and their order was counterbalanced on each day. Each day, rats in two groups (Paired-Mock and Paired-mKal7) were administered amphetamine (1.5 mg/kg, IP) in the open field and saline (1.0 ml/kg, IP) in the home cage. Rats in two additional groups (Unpaired-Mock and Unpaired-mKal7) were administered the same

injections but in the reverse location: saline in the open field and amphetamine in the home cage. Rats in two final groups (Control-Mock and Control-mKal7) received saline in both environments. Locomotor activity was recorded for two hours following injections in the open field.

Following the 4 days of exposure, rats were left undisturbed in the home cage for 1 week of withdrawal. Rats were then tested for conditioned locomotion for 1-hour following a saline injection (1.0 ml/kg, IP). Five days later, the same rats were tested for locomotor sensitization for 2-hours following an amphetamine injection (1.0 mg/kg, IP). Thus, six separate groups of rats were tested in this experiment (Table 2).

**Viral Vectors and Microinjections**

Replication-deficient HSV vectors (p1005) were constructed as described previously (Neve *et al*, 1997). The average titer of the viral stocks used was $4.0 \times 10^7$ infectious units/ml. pEAK10.His.Myc Kal7 T1590A, a threonine-alanine mutant construct of Kal7 [mKal7(T1590A)] that is not phosphorylated by Cdk5, was generously supplied by Dr. Betty Eipper (University of Connecticut, Farmington, CT) and packaged into the HSV vectors by Dr. Rachael Neve (Massachusetts Institute of Technology, Cambridge, MA). Construct empty HSV-GFP (p1005) vectors were also provided by Dr. Neve. mKal7 was driven by the HSV IE 4/5 promoter and GFP by a CMV promoter. As expected, the HSV vectors produced a transient increase in transgene expression that was observed 4 days and had dissipated 8 days post-infection (Carlezon *et al*, 1997; Neve *et al*, 1997). mKal7 infected rats showed a significant increase in Kalirin protein expression in the NAcc (146.0±15.0%) compared to controls (100±2.7%) at day 4 post-infection ($t_6$=1.944, $p<0.05$). No increase was detectable 8 days post infection.

Even at the time of maximal mKal7 expression, it is likely that endogenous Kal7 remained in the NAcc rendering the serine-alanine non-phosphorylating Kal7 mutant a dominant negative that interfered with endogenous kinase function. Dominant-negative effects can be exerted by competition with endogenous kinase for upstream activators, downstream substrates, or subcellular regulatory pathways. Thus, it is possible that endogenous Kal7 was down-regulated by a cellular negative feedback mechanism or that it was out-competed directly by mKal7 for activators and substrates. Alternatively, mKal7 may have also achieved the latter effects indirectly by occupying postsynaptic density (PSD) anchoring substrates such as PSD-95 (Penzes *et al*, 2001) that normally bind endogenous Kal7, thereby reducing PSD Kal7 levels available for Cdk5 phosphorylation.

After recovery from surgery, rats were transferred to a biosafety level 2 facility where they were administered bilateral infusions into the NAcc of HSV-mKal7 or control infusions of HSV-GFP or the 1X PBS vehicle (Mock). The mKal7 was infused as a 1:10 dilution of stock HSV-mKal7 in sterile 1X PBS. The latter two control infusions were used interchangeably as they did not differ in their behavioral effects. Microinjections were made in freely moving rats in a volume of 2 µl/side over 10 minutes through 28 gauge cannulae extending 4 mm beyond the guide cannula tips. Injection cannulae were connected via PE20 tubing to 10 µl syringes (Hamilton, Reno, NV) and left in place for 5 minutes after the injection to allow for diffusion. Rats were returned to the colony room the following day and the drug exposure phase initiated.

**Immunofluorescence**

Immunofluorescence was used in separate rats administered HSV-mKal7 or HSV-GFP to visualize the distribution of infected cells around the injection cannula tips (Figure 4c). Rats were anesthetized with ketamine (100 mg/kg, IP) and xylazine (10 mg/kg, IP) and perfused with saline followed by 4% paraformaldehyde (PF). Brains were harvested, stored in 4% PF for 48 hours, and transferred to a 25% sucrose solution for at least an additional 48 hours. 40 µm coronal sections were then obtained using a cryostat and transferred to 1X PBS for free-floating immunohistochemistry. Slices were washed multiple times with 0.3% Tween20 solution, blocked in 10% normal donkey solution (Jackson ImmunoResearch Laboratories, West Grove, PA), and then incubated overnight in this solution containing a GFP antibody fused to FITC (1:400, Abcam #ab6662) to amplify fluorescence. The next day, slices were again washed in 1X PBS and then mounted onto gelatin-coated slides and cover slipped for imaging. Examination of consecutive brain sections revealed that GFP-positive neurons were observed only in close proximity to the injection cannula tips in the NAcc shell (Figure 4c).

**Immunoblotting**

The extent of overexpression of Kal7 was determined with immunoblotting in separate rats administered HSV-mKal7 (n=4) or HSV-GFP (n=4). Brains were removed rapidly, sections (1 mm thick) obtained with a brain matrix, and 2-mm-diameter punches taken bilaterally around the injection cannula tips. Detailed procedures for immunoblotting were as described previously (Loweth *et al*, 2010; Singer *et al*, 2010). Briefly, following transfer and incubation in blocking solution, membranes were incubated with a primary antibody for Kalirin (1:500; Sigma-Aldrich, St Louis, MO) or Tubulin (1:10 000; Santa

Cruz Biotechnology, Santa Cruz, CA). Membranes were then incubated in a horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG and visualized using the ECL detection system (ECL Advanced; GE Healthcare, Waukesha, WI).

**Histological Verification of Cannula Tip Placements**

At the conclusion of the behavioral experiments, rats were anesthetized with ketamine (100 mg/kg, IP) and xylazine (10 mg/kg, IP) and perfused intracardially with 0.9% saline followed by 10% formalin for the roscovitine experiment or 4% PF for the HSV-mKal7 experiment. Brains were then harvested, stored in either 10% formalin or 4% PF for approximately 1 week, and 40 µm coronal slices subsequently obtained with a cryostat. Brain slices were mounted on gelatin-coated slides and stained with cresyl violet to verify cannula tip placements (Figure 2c). Only rats with bilateral cannula tips placed correctly in the NAcc shell were included in the behavioral analyses (Figures 2c and 4c). No consistent evidence for DMSO-induced toxicity at the injection cannula tips was detected.

**Statistical Analyses**

The session total locomotor counts obtained during drug exposure were analyzed using three-way (two between one within) ANOVA with conditioning group and either roscovitine or HSV infection as the between factors and days as the within factor. Locomotor counts obtained on the tests for conditioning and sensitization were similarly analyzed with three-way (two between one within) ANOVA but with time as the within factor. Only statistically significant effects and interactions are reported. Post hoc comparisons were conducted using the LSD test. Immunoblotting data measuring Kal7 expression were analyzed using the t-test.

## RESULTS

### Effect of Inhibiting cdk5 in the NAcc on the Induction of Locomotor Conditioning and Sensitization by Amphetamine

As expected, Paired rats administered amphetamine in the open field during the exposure phase displayed a greater locomotor response than Unpaired and Control rats administered saline. Like previous reports with cocaine (Bibb *et al*, 2001; Taylor *et al*, 2007), roscovitine spared the increase in amphetamine-induced locomotion over days although, unlike these reports, it did not enhance it (Figure 1). The ANOVA conducted on these data revealed a significant effect of conditioning ($F(2,63)=67.75$, $p<0.001$) and a significant conditioning X day interaction ($F(6,189)=4.97$, $p<0.001$). The significant interaction reflects the finding indicated by posthoc comparisons that rats in both the Paired-Veh and Paired-Ros groups showed significant increases in locomotion over days while the opposite was true for rats in the remaining groups. No significant differences were detected between the Paired-Veh and Paired-Ros groups on any day.

Again as expected, repeated pairings of amphetamine and the open field in Paired-Veh rats led to a significant conditioned locomotor response relative to Unpaired-Veh and Control-Veh rats on the conditioning test conducted one week later. Roscovitine administered to the NAcc before each amphetamine injection during exposure blocked the induction of this conditioning as exemplified by the lack of a conditioned locomotor response in Paired-Ros rats on the test (Figure 2a). The ANOVA conducted on these data revealed significant effects of conditioning ($F(2,31)=4.31$, $p<0.05$) and time ($F(5,155)=146.98$, $p<0.001$) as well as significant conditioning X time

(F(10,155)=4.00, $p$<0.001) and roscovitine X time (F(5,155)=5.39, $p$<0.001) interactions. Post-hoc LSD tests revealed that Paired-Veh rats displayed significantly greater locomotor responding overall compared to all other groups on this test ($p$<0.01-0.05). The remaining groups did not differ significantly from each other.

Unlike the effect on conditioning, roscovitine administered prior to the amphetamine injections during exposure had no effect on the induction of locomotor sensitization. On the test for sensitization also conducted one week after the last drug exposure injection, Paired-Veh and Paired-Ros rats showed a similar and significantly greater locomotor response than Unpaired and Control rats (Figure 2b). The lack of a sensitized locomotor response in Unpaired-Veh relative to Control-Veh rats is consistent with previous reports of context-specific sensitization, an effect thought to depend on conditioned inhibition of the expression of sensitization by drug-unpaired cues (Anagnostaras *et al*, 2002; Stewart and Vezina, 1991; Vezina and Leyton, 2009). Cdk5 inhibition did not affect the accrual of this type of learning as Unpaired-Ros rats similarly did not exhibit enhanced locomotion on the test for sensitization. The ANOVA conducted on these data revealed a significant effect of conditioning (F(2,29)=15.71, $p$<0.001) and time (F(11,319)=94.98, $p$<0.001) as well as a significant conditioning X time interaction (F(22,319)=6.14, $p$<0.001). Post-hoc LSD tests showed that rats in the Paired-Ros and Paired-Veh groups did not differ significantly from each other but did differ significantly from all remaining groups ($p$<0.05-0.001). These remaining groups did not differ significantly from one another.

**Effect of Transiently Expressing mKal7 in the NAcc on the Induction of Locomotor Conditioning and Sensitization by Amphetamine**

Paired rats administered amphetamine in the open field during the exposure phase again displayed a greater locomotor response than Unpaired and Control rats administered saline. As in the roscovitine experiment, the amphetamine-induced locomotion increased over days and this effect was spared though not enhanced by mKal7 expression in the NAcc (Figure 3). The ANOVA conducted on these data revealed significant effects of conditioning ($F(2,25)=50.66$, $p<0.001$) and day ($F(3,75)=6.14$, $p<0.001$) as well as significant conditioning X day ($F(6,75)=12.34$, $p<0.001$) and conditioning X day X infection ($F(6,75)=2.59$, $p<0.05$) interactions. Post hoc comparisons indicated that, unlike the remaining groups, rats in both the Paired-Mock and Paired-mKal7 groups showed significant increases in locomotion over days. No significant differences were detected between these two groups on any day.

In a manner strikingly similar to the results obtained with roscovitine, transient expression of mKal7 in the NAcc exclusively during exposure prevented the induction of locomotor conditioning as evidenced by the lack of a conditioned response in Paired-mKal7 rats on the test for conditioning conducted one week after exposure when mKal7 was no longer expressed (Figure 4a). The ANOVA conducted on these data revealed significant effects of conditioning ($F(2,25)=7.55$, $p<0.01$) and time ($F(5,125)=102.74$, $p<0.001$) as well as significant conditioning X infection ($F(2,25)=3.89$, $p<0.05$) and conditioning X time ($F(10,125)=2.31$, $p<0.05$) interactions. Post-hoc LSD tests showed that Paired-Mock rats displayed significantly greater locomotor responding on this test compared to all other groups ($p<0.05$). These did not differ significantly from each other.

Again in a manner strikingly similar to what was observed with roscovitine, transient expression of mKal7 in the NAcc during exposure did not affect the induction

of locomotor sensitization. On the subsequent test for sensitization conducted when mKal7 was no longer expressed, Paired-Mock and Paired-mKal7 rats displayed a similar and significantly greater locomotor response than Unpaired and Control rats (Figure 4b). In addition, as with roscovitine, mKal7 expression during exposure did not affect the accrual of associations between contextual cues and the absence of amphetamine. Like Unpaired-Mock rats, Unpaired-mKal7 rats also did not exhibit enhanced locomotion on the test for sensitization. The ANOVA conducted on these data revealed significant effects of conditioning ($F(2,25)=5.84$, $p<0.01$) and time ($F(11,275)=90.76$, $p<0.01$) as well as a significant conditioning X time interaction. Post-hoc LSD tests showed that rats in the Paired-mKal7 and Paired-Mock groups did not differ from each other but did differ significantly from rats in the remaining groups ($p<0.01$). These did not differ significantly from one another.

## DISCUSSION

The present findings show that preventing the phosphorylation of Kal7 by Cdk5 in the NAcc exclusively during exposure to amphetamine prevents the development of conditioned locomotion but spares the development of locomotor sensitization by the drug. These results indicate that Cdk5 phosphorylation of Kal7 in the NAcc is necessary for the induction of excitatory contextual associative conditioning but not for non-associative forms of plasticity such as sensitization.

These findings follow and are consistent with others showing that pharmacological inhibition of Cdk5 and knock out of the *Kal7* gene both prevent psychostimulant-induced increases in dendritic spine density in the NAcc but spare the

induction of locomotor sensitization (Bibb *et al*, 2001; Norrholm *et al*, 2003; Taylor *et al*, 2007) as well as others showing that amphetamine-induced changes in dendritic morphology in the NAcc correspond to associative drug conditioning rather than nonassociative drug sensitization (Singer *et al*, 2009). Considering the importance of dendritic spine proliferation in learning (Geinisman et al, 2001; Leuner et al, 2003; Lamprecht and LeDoux, 2004), these findings together suggest that Cdk5 inhibition and mKal7 expression in the NAcc prevented the induction of locomotor conditioning in the present experiments by preventing the neuroadaptations necessary to regulate spine dynamics in this site.

The present experiments assessed, in an anatomically and temporally specific manner, the contribution of Cdk5 and Kal7 to the induction of amphetamine-induced locomotor conditioning and sensitization by using a pharmacological Cdk5 inhibitor or a transient viral infection system to express mKal7 specifically in the NAcc and exclusively during amphetamine exposure. This permitted the unambiguous interpretation of the results obtained on the conditioning and sensitization tests as these were conducted days after dissipation of the pharmacological challenge and mutant protein expression: Cdk5 phosphorylation of Kal7 in the NAcc is necessary for the induction of conditioned locomotion but not locomotor sensitization by amphetamine. In contrast, conflicting results have been described in a number of recent reports using knock-out, knock-down or transgenic mice as well as long-lasting lentiviral-mediated gene transfer to manipulate Cdk5 and Kal7, possibly because these manipulations spanned the induction and expression phases of conditioning and could not distinguish between the two. Thus, in these experiments, decreasing Cdk5 or Kal7 activity produced either no

change (Benavides *et al*, 2007; Wang *et al*, 2013), a decrease (Kiraly *et* al, 2010), an increase (Hawasli *et al*, 2007), or both a decrease and an increase in conditioning (Benevides et al, 2007) while increasing Cdk5 activity increased conditioning (Fischer *et al*, 2005). A number of procedural differences between these different studies may have contributed to the different results obtained, including the subnucleus of the NAcc targeted for study (core: Benavides *et al*, 2007; Kiraly *et al*, 2010; Wang *et al*, 2013; cf, Bibb *et al*, 2001, core; Norrholm *et al*, 2003 and Taylor *et al*, 2007, shell), the type of conditioning assayed (appetitive: Benavides *et al*, 2007; Kiraly *et al*, 2010; fear: Fischer *et al*, 2005; Hawasli *et al*, 2007), and whether instrumental (Wang *et al*, 2013) as opposed to Pavlovian conditioning was tested. However, it is also possible that the different neuronal events underlying the induction and expression of conditioning were differentially affected by changes in Cdk5 and Kal7 in these experiments, rendering the effects ultimately observed difficult to interpret. Supporting this possibility, we recently found that, unlike the results obtained in the present experiments, inhibiting NAcc Cdk5 not during exposure but immediately before testing enhanced the expression of amphetamine-induced locomotor conditioning and sensitization (Singer *et al*, in review).

As expected, the locomotor sensitization observed in the present experiments was context-specific, observed in Paired but not Unpaired rats, an effect thought to depend on conditioned inhibition of the expression of sensitization by drug-unpaired cues in the latter rats (Anagnostaras et al, 2002; Stewart and Vezina, 1991; Vezina and Leyton, 2009). Interestingly, neither Cdk5 inhibition nor mKal expression in the NAcc during exposure affected this type of learning as expression of sensitization in these animals remained inhibited relative to rats in the Paired groups. These results suggest

that phosphorylation of Kal7 by Cdk5 in the NAcc is not necessary for the accrual of associations between contextual cues and the absence of amphetamine but rather is specific to the formation of excitatory associations between these cues and the presence of the drug. While both associative in nature, excitatory conditioning and conditioned inhibition reflect different contingencies, produce different behavioral effects, are regulated differently (for a review and discussion, see Vezina and Leyton, 2009), and as suggested by the present results, are likely mediated by different neuronal mechanisms.

The nature of the neuroadaptations underlying the induction of excitatory associative conditioning in the NAcc remains unknown. Changes in spines and dendritic morphology have been proposed to embody the neural representation of memory (Koleske, 2013; Lamprecht and LeDoux, 2004). According to this possibility, repeated psychostimulant exposure could increase Cdk5 in the NAcc via long-lasting increases in ΔFosB, a transcription factor for this protein (Hope *et al*, 1994), and lead to phosphorylation of Kal7 and other proteins (Barnett and Bibb, 2011) to produce stable changes in dendritic spine morphology. However, it is unlikely that such a static drug-induced change in dendritic spines mediated the excitatory associative conditioning observed in the present experiments as Paired and Unpaired rats were exposed to the same number of amphetamine exposure injections but only Paired rats showed a conditioned response when tested in the drug-paired context. Rather, the results reported here suggest that Cdk5 phosphorylation of Kal7, and perhaps other proteins, leads to neuroadaptations necessary for drug-paired cues, when present, to evoke changes in dendritic spines in the NAcc. In support of this possibility, rapid increases in

spine tip diameter were recently reported in the NAcc following presentation of cocaine-paired cues (Gipson *et al*, 2013).

**ACKNOWLEDGEMENTS**

This research was funded by National Institutes of Health grants R01 DA09397 (PV), T32 DA07255 (BFS, NMN, KRR), and F31 DA030021-01A1 (BFS). This work was also partially funded by the Chicago Biomedical Consortium with support from The Searle Funds at The Chicago Community Trust (BFS).

**DISCLOSURE**

The authors declare no conflict of interest.

## FIGURE LEGENDS

**Figure 1.** Locomotor activity observed during the exposure phase: NAcc roscovitine. Data are shown as mean (+SEM) two-hour total locomotor counts observed following amphetamine (Paired) or saline (Unpaired and Control) injections on each of the four exposure days. NAcc roscovitine (Ros) produced no detectable effects. n/group=5-7.

**Figure 2.** NAcc roscovitine (Ros) administered during exposure blocked the induction of conditioned locomotion but spared the induction of locomotor sensitization. Time course (left) and session total locomotor counts (right) are shown for the one-hour conditioning test (A) and two-hour sensitization test (B). Data are shown as mean (±SEM). *****, $p<0.05$, compared to all other groups. †, $p<0.05$, either Paired-Veh or Paired-Ros compared to remaining groups. C. Line drawings (Paxinos and Watson, 1997) depicting location of microinjection cannula tips in the NAcc shell for rats included in the data analyses (left). Numbers indicate mm from bregma. The photomicrograph to the right shows a representative cresyl violet stained brain section with bilateral cannula tracks targeting the NAcc shell. n/group=5-7.

**Figure 3.** Locomotor activity observed during the exposure phase: NAcc mKal7. Data are shown as mean (+SEM) two-hour total locomotor counts observed following amphetamine (Paired) or saline (Unpaired and Control) injections on each of the four exposure days. NAcc mKal7 produced no detectable effects. n/group=5-6.

**Figure 4.** Transient expression of mKal7 in the NAcc during exposure blocked the induction of conditioned locomotion but spared the induction of locomotor sensitization. Time course (left) and session total locomotor counts (right) are shown for the one-hour conditioning test (A) and two-hour sensitization test (B). Data are shown as mean (±SEM). *****, $p<0.05$, compared to all other groups. ******, $p<0.01$, either Paired-Veh or Paired-Ros compared to remaining groups. C. Line drawings (Paxinos and Watson, 1997) depicting location of microinjection cannula tips in the NAcc shell for rats included in the data analyses (left). Numbers indicate mm from bregma. The photomicrograph to the right was obtained 4 days after infection with HSV-mKal7(T1590A)-GFP and illustrates GFP-positive neurons in close proximity to the injection cannula tip in the NAcc shell (arrow). n/group=5-6.

## REFERENCES


Anagnostaras SG, Robinson TE (1996). Sensitization to the psychomotor stimulant effects of amphetamine: Modulation by associative learning. *Behavioral Neuroscience* **110**: 1397–1414.

Anagnostaras SG, Schallert T, Robinson TE (2002). Memory processes governing amphetamine-induced psychomotor sensitization. *Neuropsychopharmacology* **26**: 703–715.

Aujla H, Beninger RJ (2004). Intra-BLA or intra-NAc infusions of the dopamine $D_3$ receptor partial agonist, BP 897, block intra-NAc amphetamine conditioned activity. *Behavioral Neuroscience* **118**: 1324-1330.

Banasikowski TJ, Bespalov A, Drescher K, Behl B, Unger L, Haupt A *et al* (2010). Double dissociation of the effects of haloperidol and the dopamine D3 receptor antagonist ABT-127 on acquisition vs. expression of coaine-conditioned activity in rats. *Journal of Pharmacology and Experimental Therapeutics* **335**: 506-515.

Barnett DGS, Bibb JA (2011). The role of Cdk5 in cognition and neuropsychiatric and neurological pathology. *Brain Research Bulletin* **85**: 9-13.

Benavides DR, Quinn JJ, Zhong P, Hawasli AH, DiLeone RJ, Kansy JW, *et al* (2007). Cdk5 modulates cocaine reward, motivation, and striatal neuron excitability. *Journal of neuroscience* **27**: 12967-12976.

Bibb JA, Chen J, Taylor JR, Svenningsson P, Nishi A, Snyder GL, *et al* (2001). Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5. *Nature* **410**: 376-380.

Carlezon WA Jr, Boundy VA, Haile CN, Lane SB, Kalb RG, Neve RL *et al* (1997). Sensitization to morphine induced by viral-mediated gene transfer. *Science* **277**: 812-814.

Carlezon WA, Neve RL (2003). Viral-mediated gene transfer to study the behavioral correlates of CREB function in the nucleus accumbens of rats. *Methods in Molecular Medicine* **79**: 331-350.

Cervo L, Samanin R (1995). Effects of dopaminergic and glutamatergic receptor antagonists on the acquisition and expression of cocaine conditioned place preference. *Brain Research* **673**: 242-250.

Crombag HS, Mueller H, Browman KE, Badiani A, Robinson TE (1999). A comparison of two behavioral measures of psychomotor activation following intravenous amphetamine or cocaine: Dose- and sensitization-dependent changes. *Behavioral Pharmacology* **10**: 205-213.

Fischer A, Sananbenesi F, Schrick C, Spiess J, Radulovic J (2002). Cyclin-dependent kinase 5 is required for associative learning. *Journal of Neuroscience* **22**: 3700-3707.

Fischer A, Sananbenesi F, Pang PT, Lu B, Tsai L-H (2005). Opposing roles of transient and prolonged expression of p25 in synaptic plasticity and hippocampus-dependent memory. *Neuron* **48**: 825-838.

Geinisman Y, Berry RW, Disterhoft JF, Power JM, Ven der Zee EA (2001). Associative learning elicits the formation of multiple-synapse boutons. *Journal of Neuroscience* **21**: 5568-5573.

Gipson CD, Kupchik YM, Shen H, Reissner KJ, Thomas CA, Kalivas PW (2013). Relapse induced by cues predicting cocaine depends on rapid, transient synaptic potentiation. *Neuron* **77**: 867-872.

Hawasli AH, Benavides DR, Nguyen C, Kansy JW, Hayashi K, Chambon P, *et al* (2007). Cyclin-dependent kinase 5 governs learning and synaptic plasticity via control of NMDAR degradation. *Nature Neuroscience* **10**: 880-886.

Hope BT, Nye HE, Kelz MB, Self DW, Iadarola MJ, Nakabeppu Y, *et al* (1994). Induction of a long-lasting AP-1 complex composed of altered Fos-like proteins in brain by chronic cocaine and other chronic treatments. *Neuron* **13**: 1235–44.

Kiraly DD, Ma X-M, Mazzone CM, Xin X, Mains RE, Eipper BA (2010). Behavioral and morphological responses to cocaine require kalirin7. *Biological Psychiatry* **68**: 249-255.

Koleske AJ (2013). Molecular mechanisms of dendrite stability. *Nature Reviews Neuroscience* **14**: 536-550.

Lamprecht R, LeDoux J (2004). Structural plasticity and memory. *Nature Reviews Neuroscience* **5**: 45–54.

Leuner B, Falduto J, Shors TJ (2003). Associative memory formation increases the observation of dendritic spines in the hippocampus. *Journal of Neuroscience* **23**: 659-665.

Leyton M, Vezina P (2013). Striatal ups and downs: Their roles in vulnerability to addictions in humans. *Neuroscience and Biobehavioral Reviews* **37**: 1999-2014.

Li F, Xue Y, Wang J, Fang Q, Li Y, Zhu W, *et al* (2010). Basolateral amygdala cdk5 activity mediates consolidation and reconsolidation of memories for cocaine cues. *Journal of Neuroscience* **30**: 10351–10359.

Loweth JA, Singer BF, Baker LK, Wilke G, Inamine H, Bubula N, *et al* (2010). Transient overexpression of α-$Ca^{2+}$/calmodulin-dependent protein kinase II in the nucleus accumbens shell enhances behavioral responding to amphetamine. *Journal of Neuroscience* **30**: 939-949.

Marie N, Canestrelli C, Noble F (2012). Transfer of neuroplasticity from nucleus accumbens core to shell is required for cocaine reward. *Plos One* **7**: e30241.

Moses SN, Sutherland RJ, McDonald RJ (2002). Differential involvement of amygdala and hippocampus in responding to novel objects and contexts. *Brain Research Bulletin* **58**: 517-527.

Neve RL, Howe JR, Hong S, Kalb RG (1997). Introduction of the glutamate receptor subunit 1 into motor neurons in vitro and in vivo using a recombinant herpes simplex virus. *Neuroscience* **79**: 435-447.

Norrholm SD, Bibb JA, Nestler EJ, Ouimet CC, Taylor JR, Greengard P (2003). Cocaine-induced proliferation of dendritic spines in nucleus accumbens is dependent on the activity of cyclin-dependent kinase-5. *Neuroscience* **116**: 19-22.

Paxinos G, Watson C (1997). The Rat Brain in Stereotaxic Coordinates, 3rd Edtion. Academic Press, New York, NY.

Pellegrino LJ, Pellegrino AS, Cushman AJ (1979). A stereotaxic atlas of the rat brain. Plenum Press, New York, NY.

Penzes P, Johnson RC, Sattler R, Zhang X, Huganir RL, Kambampati V *et al* (2001). The neuronal Rho-GEF Kalirin-7 interacts with PDZ domain-containing proteins and regulates dendritic morphogenesis. *Neuron* **29**: 229-242.

Penzes P, Jones KA (2008). Dendritic spine dynamics - a key role for kalirin-7. *Trends in Neurosciences* **31**: 419-427.

Robinson TE, Kolb B (1997). Persistent structural modifications in nucleus accumbens and prefrontal cortex neurons produced by previous experience with amphetamine. *Journal of Neuroscience* **17**: 8491-8497.

Robinson TE, Kolb B (1999). Alterations in the morphology of dendrites and dendritic spines in the nucleus accumbens and prefrontal cortex following repeated treatment with amphetamine and cocaine. *European Journal of Neuroscience* **11**: 1598-1604.

Singer BF, Forneris J, Vezina P (in review). Inhibiting cyclin-dependent kinase 5 in the nucleus accumbens enhances the expression of amphetamine-induced locomotor conditioning and sensitization.

Singer BF, Loweth JA, Neve RL, Vezina P (2010). Transient viral-mediated overexpression of α-calcium/calmodulin-dependent protein kinase II in the nucleus accumbens shell leads to long-lasting functional upregulation of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors: dopamine type-1 receptor and protein kinase A dependence. *European Journal of Neuroscience* **31**: 1243-1251.

Singer BF, Tanabe LM, Gorny G, Jake-Matthews C, Li Y, Kolb B, *et al* (2009). Amphetamine-induced changes in dendritic morphology in rat forebrain correspond to associative drug conditioning rather than nonassociative drug sensitization. *Biological Psychiatry* **65**: 835–840.

Stewart J (1992). Neurobiology of conditioning to drugs of abuse. In: Kalivas PW, Samson HH (eds) *The Neurobiology of Drug and Alcohol Addiction*. New York Academy of Sciences: New York, pp 335-346.

Stewart J, Vezina P (1987). Environment-specific enhancement of the hyperactivity induced by systemic or intra-VTA morphine injections in rats pre-exposed to amphetamine. *Psychobiology* **15**: 144-153.

Stewart J, Vezina P (1988). Conditioning and behavioral sensitization. In: Kalivas PW, Barnes CD (eds) *Sensitization in the Nervous System*. Telford Press: Caldwell, NJ, pp 207-224.

Stewart J, Vezina P (1991). Extinction procedures abolish conditioned stimulus control but spare sensitized responding to amphetamine. *Behavioural pharmacology* **2**: 65–71.

Taylor JR, Lynch WJ, Sanchez H, Olausson P, Nestler EJ, Bibb JA (2007). Inhibition of Cdk5 in the nucleus accumbens enhances the locomotor-activating and incentive-motivational effects of cocaine. *Proceedings of the National Academy of Sciences* **104**: 4147-4152.

Vezina P (2004). Sensitization of midbrain dopamine neuron reactivity and the self-administration of psychomotor stimulant drugs. *Neuroscience and Biobehavioral Reviews* **27**: 827–839.

Vezina P, Leyton M (2009). Conditioned cues and the expression of stimulant sensitization in animals and humans. *Neuropharmacology* **56**: 160-168.

Vezina P, Stewart J (1990). Amphetamine administered to the ventral tegmental area but not to the nucleus accumbens sensitizes rats to systemic morphine: Lack of conditioned effects. *Brain Research* **516**: 99-106.

Wang X, Cahill ME, Werner CT, Christoffel DJ, Golden SA, Xie Z *et al* (2013). Kalirin-7 mediates cocaine-induced AMPA receptor and spine plasticity, enabling incentive sensitization. *Journal of Neuroscience* **33**: 11012-11022.

Wang Y-C, Hsiao S (2003). Amphetamine sensitization: Nonassociative and associative components. *Behavioral Neuroscience* **117**: 961–969.

Xie Z, Srivastava DP, Photowala H, Kai L, Cahill ME, Woolfrey KM, *et al* (2007). Kalirin-7 controls activity-dependent structural and functional plasticity of dendritic spines. *Neuron* **56**: 640-656.

Xin X, Wang Y, Ma X, Rompolas P, Keutmann HT, Mains RE, *et al* (2008). Regulation of Kalirin by Cdk5. *Journal of Cell Science* **121**: 2601-2611.